# The Delphion Integrated View



Title: JP5246885A2: PREVENTING AND THREAPEUTIC AGENT FOR DISE CAUSED BY CEREBRAL DYSFUNCTION
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Abstract:

PURPOSE: To obtain a preventing and therapeutic agent, having cell survival prolonging and/or acetylcholine transferase activating actions and useful for preventing and treating diseases caused by various cerebral dysfunctions.
CONSTITUTION: The objective preventing and therapeutic agent comprises one or two or more hematopoietic factors selected from an erythropoietin, a granulocyte colony stimulating factor and a macrophage colony stimulating factor as an active ingredient and is useful for diseases caused by cerebral dysfunctions. Since this agent has excellent cell survival prolonging and acetylcholine transferase(ChAT) activating actions and low side effects, it is useful as a medicine applied to various diseases caused by the cerebral dysfunctions including Alzheimer disease, Alzheimer type senile dementia or cerebrovascular dementia.


Family: None
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[Scope of Claims for Patent]

[Claim 1] A drug for preventing or curing disease due to the cerebral function disorder, which contains, as an effective ingredient, 1 or 2 or more kinds of hematopoietic factors selected from erythropoietin, granulocyte colony stimulating factor and macrophage colony stimulating factor.

[Claim 2] The drug for preventing or curing dementia due to the cerebral function disorder as defined in claim 1, wherein the disease due to the cerebral function disorder is the disease for which the cell survival prolonging activity and/or acetylcholinetransferase activating activity is effective.

[Claim 3] The drug for preventing or curing diseases due to the cerebral function disorder as defined in claim 1, wherein the disease due to the cerebral function disorder is Alzheimer's disease, Alzheimer type senile dementia or cerebrovascular dementia.

[Detailed explanation of the invention]

[0001]

[Industrial field of the applicability] The present invention relates to a therapeutic drug used for preventing or curing diseases due to the cerebral function disorder, more particularly, a drug for preventing or curing diseases due to the cerebral function disorder containing erythropoietin, granulocyte colony stimulating factor or macrophage colony stimulating factor as an effective ingredient.

[0002]

[Prior art] As diseases due to the cerebral function disorder,

there are known Alzheimer's disease which is hereditary and is rapid in progress, Alzheimer type senile dementia which is developed at an old age and is non-hereditary and slow in progress, and cerebrovascular dementia such as memory disorder, mental disorder, volition decrease and aprosexia which are thought to be associated with cerebral ischemia disorder such as cerebral infarct and encephalorrhagy, and cerebral circulation disorder. Further, in these diseases, symptom of memory disorder appears remarkably as their great characteristics at an initial phase of attack, and this is considered to be due to comparatively selective degeneration and shedding of cholinergic nerve cells of cerebral basal ganglia at an initial stage of progress of diseases. Such the diseases due to the cerebral function disorder is becoming a social problem while an aged population is rapidly increased recently and also in the pharmaceutical buisiness, there is urgently demanded development of therapeutic drug for fundamentally preventing or curing these diseases.

[0003] Then, previously, the mechanism of attack of such the diseases and development of therapeutic drugs therefor have been variously studied, and development of some therapeutics have been tried. For example, since Alzheimer type senile dementia is associated with decrease in the function of intracerebral cholinergic nerve system, i.e., decrease in an amount of intracerebral acetylcholine, in order to increase an amount of this intracerebral acetylcholine, there has been provided use of an acetylcholinesterase inhibiting agent for inhibiting the activity of an acetylcholine precursor or

acetylcholinesterase which is an acetylcholine degrading enzyme, and actually there has been proposed use of physostigmine, tetrahydroaminoacridine and the like as an acetylcholinesterase inhibiting agent. However, these drugs have the insufficient therapeutic efficacy for disease due to cerebral function disorder including Alzheimer type senile dementia, and further, there is a problem of unfavorable side effects.

[0004] In addition, recently, there has been proposed use of a substance having the acetylcholinetransferase activating activity (ChAT activating activity) for activating the activity of acethylcholinetransferase (ChAT) which is an acethylcholine synthesizing enzyme, for the purpose of increasing an amount of intracerebral acetylcholine as described above but not utilizing the above-mentioned acetylcholinesterase inhibiting activity, and actually, there have been reported a drug for curing or preventing senile dementia containing interleukin 3(IL-3) as an active ingredient [JP-A No. 3-93728, Kamegai et al., Neuron, 4, 429-436 (March 1990), Kamegai et al., Cerebral Research, 532, 323-325(1990)]. Further it has been reported that nerve growth factor (NGF) acts on sympathetic nerve, sensory nerve or forebrain cholinergic nerve cells to promote their differentiation and maturation and are effective in survival and function maintenance [Dev. Brain Res. 9, 45-52(1983)], and that Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) has the in vitro activity as a neural nutrition factor [Kamegai et al Brain Research, 532, 323-325 (1990)].

[0005]

[Problems to be solved by the invention] The present inventors noted the fact that diseases due to the cerebral function disorder are accompanied with symptom of memory disorder as characteristics thereof at an early stage of attack, and cholinergic nerve cells of cerebral basal ganglia are comparatively selectively denatured and shed. Further the have intensively studied development of a therapeutic which can prevent or treat denaturation and shedding of the cholinergic nerve cells and, as a result, found that erythropoietin (EPO) which is a hematopoietic factor, granulocyte colony stimulating factor (G-CSF) and macrophage colony stimulating factor (M-CSF) have the excellent cell survival prolonging action and acetylcholinetransferase activating action, and are effective for preventing and curing diseases due to the cerebral function disorder, which resulted in completion of the present invention.

[0006] Accordingly, an object of the present invention is to provide a preventing and curing drug which is effective for preventing and curing various disease due to the cerebral function disorder. In addition, an object of the present invention is to provide a preventing or curing drug which is effective for preventing and curing diseases for which the cell survival prolonging action and/or acetylcholinetransferase activating action are effective. Further, an object of the present invention is to provide a preventing and curing drug which is effective for preventing and curing Alzheimer's disease, Alzheimer type senile dementia or cerebrovascular dementia.

[0007]

[Means to solve the problems] The present invention is a drug for preventing or curing diseases due to the cerebral function disorder, which contains, as an effective ingredient, one or two or more kinds of hematopoietic factors selected from erythropoietin, granulocyte colony stimulating factor and macrophage colony stimulating factor.

[0008] It is contemplated that erythropoietin, granulocyte colony stimulating factor and macrophage colony stimulating factor of a hematopoietic factor which are used as an active ingredient in the present invention may be ones prepared by any method as far as they exert the essential activity as a hematopoietic factor. That is, they may be hematopoietic factors which are extracted from the nature, or prepared by the genetic recombination techniques. Upon this, the transformant cell may be either of prokaryote or eukaryote.

[0009] That is, examples of erythropoietin (EPO) include natural human EPO obtained by extracting urine of human aplastic anemia patient (JP-B No. 1-38800), EPO prepared by the genetic recombination techniques consisting of the steps: collecting a messenger RNA (mRNA) corresponding to the amino acid sequence of human EPO, preparing a recombinant DNA utilizing the mRNA and, then, producing EPO in a suitable host (e.g. bacteria such as Escherichia coli, yeasts, plant cell strains, animal cells such as COS cell, Chinese hamster ovary cell (CHO), mouse C-127 cell etc.) [e.g. JP-B No. 1-44317, Kenneth Jcops et al, Nature, 313, 806-810(1985)] and the like. Further, specifically, there can be mentioned natural human urine EPO, and EPO such as rhEPO/CHO

which is genetic recombinant human EPO produced by utilizing Chinese hamster ovary cell (CHO) as a host.

[0010] In addition, examples of granulocyte colony stimulating factor (G-CSF) include natural human G-CSF obtained by culturing human G-CSF producing cell, and extracting, separating and purifying the culture supernatant (JP-B No. 1-44200), G-CSF obtained by transforming a host such as Escherichia coli and animal cells by genetic recombination to obtain a transformant, and then isolating and purifying the transformant, or chemical modification thereof (e.g. JP-B No. 2-5395, JP-A No. 62-129298, JP-A No. 62-132899, JP-A No. 62-236488, JP-A No. 64-85098). Further, specifically, there are natural human G-CSF, rhG-CSF-CHO which is genetic recombinant human G-CSF produced using Chinese hamster ovary cell (CHO) as a host, and rhG-CSF E. coli. which is genetic recombinant human G-CSF produced using E coli. as a host.

[0011] Further, examples of macrophage colony stimulating factor (M-CSF) include natural human M-CSF obtained by extracting, separating and purifying a biological sample such as human urine (e.g. JP-A No. 64-22899, JP-A No. 3-17021), and M-CSF prepared by the genetic recombination techniques (e.g. JP-A No. 62-501607, JP-A No. 1-502397). Further, specifically, there can be mentioned natural human M-CSF, rhM-CSF/CHO which is genetic recombinant human M-CSF produced using Chinese hamster ovary cell (CHO) as a host and rhM-CSF/E. coli. which is genetic recombinant human M-CSF produced using E. coli. as a host.

[0012] As a route for administering such the EPO, G-CSF

or M-CSF, there can be contemplated intracerebral administration for directly administering a drug into a cerebral surgically, and intra-cerebrospinal fluid administration for directly injecting a drug into a cerebrospinal fluid. Further intravenous injection is expected to be possible.

[0013] Further, a dose of the EPO, G-CSF or M-CSF can be appropriately determined in consideration of disease to be targeted and conditions of the diseases and, in the case of EPO, a dose is usually 0.1 to 500 μg, preferably 5 to 100 μg per adult and, in the case of G-CSF, a dose is usually 0.1 to 1,000 μg, preferably 1 to 700 μg per adult and, in the case of M-CSF, a dose is usually 0.1 to 1,000 μg, preferably 1 to 700 μg per adult.

[0014] Further, a suspending agent, a solubilizer, a stabilizing agent, an isotonic agent, a preservative and an adsorption preventing agent may be added to the preparation containing EPO, G-CSF or M-CSF as an effective ingredient of the present invention, if necessary depending on an administration method and a dosage form. Examples of the suspending agent include methylcellulose, Polysorbate 80, hydroxyethylcellulose, gum arabic, tragacanth powder, sodium carboxymethylcellulose and polyoxyethylene sorbitan monolaurate. Examples of the solubilizer include polyoxyethylene hardened castor oil, Polysorbate 80, nicotinic acid amide, polyoxyethylene sorbitan monolaurate, macrogol and castor oil fatty acid ethyl ester. Examples of the stabilizing agent include serum albumin, dextran 40, methylcellulose, gelatin, sodium sulfite and sodium metasulfite. Examples of

the isotonic agent include D-mannitol and sorbitol. Examples of the preservative include methyl paraoxybenzoate, ethyl paraoxybenzoate, sorbic acid, phenol, cresol and chlorocresol. Further, examples of the adsorption preventing agent include human serum albumin, lecithin, dextran, ethylene oxide/propylene oxide copolymer, hydroxypropylcellulose, methylcellulose, polyoxyethylene hardened castor oil and polyethylene glycol.

[0015] Experimental examples for confirming the effects of the invention will be described below.

[1] Preparation of primary cultured cell

Septal area containing forebrain base was obtained from BALB/mouse (Sankyo experimental Service, Tokyo) at a fatal phase 15 days. The tissue was isolated and cut finely in Hanks' balanced salt solution (HBSS solution, pH7.4), and was treated with the HBSS solution (pH6.5) containing 0.03% trypsin at 37°C for 3 minutes. This was filtered with 63 μm nylon mesh, and resuspended in a serum-free medium. Culturing was started at $6 \times 10^5$ cells/ml. At initiation of cell culturing, 100ng/ml of mouse βNGF (mβNGF, Sigma, Missouri), 10CFU/ml, 50CFU/ml or 100CFU/ml of recombinant human macrophage colony stimulating factor (rhM-CSF) (Genzyme, Massachusetts, USA), 10CFU/ml, 50CFU/ml or 100CFU/ml of recombinant human granulocyte colony stimulating factor (rhG-GSF) (manufactured by Chugai Pharmaceutical Co., Ltd.), or 1IU/ml, 5IU/ml or 10IU/ml of recombinant human erythropoietin (rhEPO)(manufactured by Chugai Pharmaceutical Co., Ltd.) was added, respectively, and

culturing solution exchange was performed after three days, and subsequently cells were recovered with a rubber policeman (equipment in which a rubber is fitted into a tip of a glass tube) at 5 day, and then the cholineacetyltransferase (ChAT) activity was measured by the following method. The results are shown in Table 1. The serum-free medium was a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) (manufactured by Gibco, New York, USA) containing 4.5g/l of glucose and Ham's F12 (manufactured by Gibco) (pH 7.4) supplemented with the following ingredients: i.e., a mixture of 15mM HEPES buffer solution, 30nM sodium selenate, a 1% penicillin-streptomycin solution (manufactured by Gibco), 100 μg/ml human transferrin, 25 μ/ml of bovine crystallized insulin, 20nM progestrone, 20nM hydrocortisone-21-phosphate, 10mM L-carnitine, 30nM 3,3',5-triiodo-L-thyronine, 7ng/ml tocopherol, 7ng/ml retinol, 1μM thioctic acid, and 1μl/ml mineral mixture [Hutchings et al., P.N.A.S., 75, 901-904 (1978)]. Unless indicated otherwise, compounds manufactured by Sigma (Louisiana or Missouri) were used.

[0016][2] Preparation of cultured cell of SN6.10.2.2

A preparation of cultured cell of SN6.10.2.2 is stored in Chicago University, USA, and the SN6.10.2.2 cell supplied by Dr. Wainer of this university is a subclone of SN6 cell [Hanmond et al., Science, 234, 1237-1240(1986)] established as a fused hybrid cell of mouse septal area nerve cell and mouse neurofibroma N18TG2. The cell was kept in DMEM containing 10% bovine fetal serum. Prior to use, $2 \times 10^5$ cells/ml were washed with a HBSS

solution (pH7.4) twice and a serum-free medium once. After cultured for 2 days in a 35mm dish (manufactured by Becton Dickinson, New Jersey, USA) in a test medium containing rhM-CSF, rhG-CSF or rhEPO, the cells were recovered with a rubber policeman on 3 day, and the ChAT activity was measured by the following method. The results are shown in Table 2.

[0017] [3] Preparation of intracerebral in vivo model by Fimbria-Fornix cutting

Wister line albino male rat (CLEA Japan, Inc.), weighing 300-350g, of an intracerebral in vivo model was anesthetized with 30 to 50mg/kg sodium pentobarbital, and a head was fixed with a cerebral stereotactic instrument (Narishigekikai, Tokyo). Then, Fimbria-Fornix cutting was performed by the following procedures; i.e., a 3mm-square part having two orthogonal sides of a line 0.5mm behind from Bregma of a left cranial bone and a median line was excised, and left back side Fimbria and Fornix together with cortex were suction-removed. A position 0.2mm ahead from Bregma of a light cranial bone and 1mm right from a median line was perforated, and a cannula (Kunui, Tokyo) having a diameter of 1mm was inserted into this pore. Thorough this cannula, 2 doses of 125 I.U./15μl/day or 12.5 I.U./15μl/day of rhEPO were continuously administered to experimental groups consisting of three rats for 4 days, respectively. As a control group, 5μg/15μl/day β-NGF or 15μl/day physiological saline was administered under the same conditions as those described above.

[0018] 2 mg/kg of diisopropyl fluorophosphate was intramuscularly administered to a rat 14 days after operation,

and after 4 hours, the rat was perfused with 100ml of a physiological saline as well as a cooled 0.1M phosphate buffer containing 300ml of 4% paraformaldehyde and 0.1% glutaraldehyde. The brain was isolated, fixed with a 2% Zamboni solution for 4 days, and was allowed to stand in a 10% sucrose solution at 4°C overnight. A cerebral crown-like piece having a thickness of 20 μm was prepared, and stained with the modified Butcher et al.'s method [Butcher et al., Neuron, 7, 197-208(1991)]. According to the Gage et al.'s method [Gage et al, Neurosicence, 19, 241-255(1986)], the acetylcholinesterase (Ache)-positive cell, i.e., nerve cell having a minimum diameter of 12 μm which is present along a main axis of inner septal area was examined used for an image analysis software (Olympus Optical Co., Ltd. Tokyo). Regarding both septal areas on Fimbria-Fornix cutting side and opposite side of individual animals, the number of AchE-positive cells was counted, and percentage obtained by dividing the number of AchE-positive cells present on a cutting side by the number of cells present on an opposite side was regarded as a survival rate of acetylcholinergic nerve cell. The results are shown in Fig.1. Column in the figure is Mean ±1 S.D., ** denotes $p<0.01$, EPO-H denotes a 125I.U./15μl/day administered group, and EPO-L denotes a 12.5I.U./15μl/day administered group, respectively.

[0019][4] Measurement of cholineacetyltransferase (ChAT) activity and Quantitaion of protein

The cholineacetyltransferase (ChAT) activity and the protein quantitative ChAT activity were obtained based on the

Fonnum's method [F. Fonnum, J. Neurochem., 24, 407-409(1975)]. In detail, the above-mentioned primary cultured cell or SN 6.10.2.2 cultured cell was recovered with a rubber policeman, homogenized in 30μl of a 10mM-EDTA solution, and the final concentration of 0.5% (v/v) of Triton-X100 was added, which was used as an enzyme source. The enzyme reactivity solution was obtained by adding 0.2mM [1-$^{14}$C]acetyl-CoA (purchased from Amersham Japan) as a substrate, 8mM-choline bromide, and 0.1mM-physostigmine as an acetylcholinesterase inhibitor to 300mM-NaCl, 50mM sodium phosphate buffer (pH7.4), and 20mM-EDTA. 5 μm of this reactivity solution was placed into a 1.5ml microtube (manufactured by Eppendorf, Germany), 2μl of an enzyme solution sample was added, and the mixture was shaken slightly to stir them, to react at 37°C for 15 minutes. The reactivity was stopped by ice-cooling, the microtube was placed into a liquid scintillation vial, and the reactivity mixture in the vial was washed out with 5ml of a 10mM-phosphate buffer. To this vial were added 2 ml of acetonitrile containing 10mg/ml of karygnost (Sodium Tetraphenylborate) and 10ml of a toluene type scintillator, followed by slight shaking for 1 minute. After the vial was allowed to stand for 10 minutes, an amount of acetylcholine labeled with $^{14}$C was determined with a liquid scintillation counter and the ChAT activity was obtained.

[0020] In addition, quantitation of a protein was performed according to the Lowry's method [Lowry et al., J. Biol. Chem., 193, 265-275(1951)]. For this purpose, 10 μm of the above-mentioned enzyme solution sample was used. Further,

based on these values, the specific activity, the total protein amount and the total ChAT activity were calculated, respectively. Regarding the ChAT activity, the protein concentration and the survival rate, a T-test was performed.

[0021]

[Table 1]

Upper row:

ChAT specific activity

Relative ChAT specific activity [a)]

Total protein amount

Relative total protein amount [b)]

Total ChAT activity

Relative ChAT activity [d)]

(Note)

a: Relative ChAT specific activity = (ChAT specific activity of sample)/(ChAT specific activity of control) × 100

b: Relative total protein amount = (Total protein amount of sample)/(Total protein amount of Control) × 100

c: Total ChAT activity = ChAT specific activity × Total protein amount

d: Relative total ChAT activity = (Total ChAT activity of sample)/(Total ChAT activity of control) × 100

[0022]

[Table 2]

Upper row:

ChAT specific activity

Relative ChAT specific activity [a)]

Total protein amount

Relative total protein amount [b)]

Total ChAT activity

Relative ChAT activity [d)]

(Note) a, b, c and d are the same as in Table 1.

[0023] As apparent from the results shown in the above Table 1, rhM-CSF, rhG-CSF or rhEPO of the present invention was added to the primary cultured system of septal area cell at a dose shown in the Table, and the ChAT specific activity, the total protein amount and the total ChAT activity after five days were examined and, as a result, in the case of rhM-CSF, the total protein amount was increased by 25%, 44% and 15% at a dose : 10CFU/ml, 50CFU/ml and 100CFU/ml relative to a control and, in the case of rhG-CSF, the total protein amount was increased by 12%, 24% and 18% at a dose : 10CFU/ml, 50CFU/ml and 100CFU/ml relative to a control and, in the case of rhEPO, the total protein amount was increased by 31%, 25% and 38% at a dose: 1IU/ml, 5IU/ml and 10IU/ml relative to a control. These values show the results equivalent to or superior over 23% which is the effect in the case of mβNGF (100ng/ml) as an experimental control, and it was found that the hematopoietic factors exert activity similar to that of mβNGF which is a nervous nutrient factor and promote survival of the primary cultured nerve cell.

[0024] In addition, regarding the ChAT specific activity, rhM-CSF increased the activity by 17% at a dose : 50CFU/ml and 100CFU/ml, respectively, based on a control, rhG-CSF increased the activity by 1%, 25% and 14%, respectively, at a dose: 10CFU/ml,

50CFU/ml and 100CFU/ml relative to a control, and rhEPO increased the activity by 20% and 18%, respectively, at a dose : 5IU/ml and 10IU/ml based on a control. Further, regarding the total ChAT activity, rhM-CSF increased the activity by 20%, 70% and 37%, respectively, at a dose : 10CFU/ml, 50CFU/ml and 100CFU/ml based on a control, rhG-CSF increased the activity by 14%, 55% and 33%, respectively, at a dose : 10CFU/ml, 50CFU/ml and 100CFU/ml relative to a control, and rhEPO increased the activity by 19%, 49% and 62%, and respectively, at a dose: 1IU/ml, 5IU/ml and 10IU/ml based on a control. From these results, it was found that a hematopoietic factor used in the present invention, rhM-CSF, rhG-CSF or rhEPO exerts the excellent effect also in the ChAT activating activity.

[0025] Further, Table 2 shows the effect of rhM-CSF, rhG-CSF and rhEPO of the present invention on the ChAT specific activity, the total protein amount and the total ChAT activity of SN6.10.2.2 cell and, in this system, as apparent also from that there is hardly a change in the total protein amount between the case of a control and the case of addition of a hematopoietic factor, the influence only on differentiation character of hematopoietic factors can be observed alone. As apparent from the results of this experiment, rhM-CSF (50CFU/ml), rhG-CSF (50CFU/ml) and rhEPO (10IU/ml) increased the ChAT specific activity by 61%, 68% and 80%, respectively, and increased the total ChAT activity by 80%, 58% and 74%, respectively.

[0026] In addition, as shown in Fig.1, in the rhEPO administered group, a survival rate of the AchE-positive cell

in septal area of the unilaterally Fimbria-Fornix cut rat was significantly improved compared to a control group (physiological saline, 0.1% BSA). In septal area in the unilaterally Fimbria-Fornix cut rat, the in vivo effect of rhEPO on acetylcholinergic nerve cell was confirmed, and this is meaningful for considering the actual clinical strategy.

[0027]

[Activity] As apparent form the above experiment, although rhM-CSF, rhG-CSF and rhEPO of the present invention are hematopoietic factors which were originally found as having the activity on blood cell system, it was found that they have the same activity as that of mβNGF also on the central nerve cell system. That is, they remarkably increase the total protein amount and, at the same time, increase the ChAT specific activity and the total ChAT activity in the in vitro primary culture of mouse septal area nerve cell, and increase the ChAT specific activity and the total ChAT activity also in the mouse septal area-derived cholinerigic nerve cell strain SN6.10.2.2 cell. Thus, these hematopoietic factors, rhM-CSF, rhG-CSF and rhEPO exert two activitys, i.e., exert the excellent cell survival prolonging activity and the ChAT activating activity. Further, the effect supporting survival of acetylcholinergic nerve cell was recognized also in the in vivo experiment, i.e., in the Fimbria-Fornix nerve route cutting system. In this system, since supply of NGF which is produced in hippocampus and reversely carried in an axon is stopped by cutting, the acetylcholinergic nerve cell in septal area can not receive the supply, leaving

to death. Therefore, it was made clear that rhEPO has the LGF-like neurotrophic factor activity in vivo.

[0028] For this reason, rhM-CSF, rhG-CSF and rhEPO of the present invention are effective for diseases in which cholinergic nerve cell is denatured and shed leading to memory disorder, for example, Alzheimer's disease and Alzheimer type senile dementia, and additionally, are effective also for cerebrovascular dementia such as memory disorder, mental disorder, volition decrease and aprosexia which are thought to be accompanied with cerebral ischemia disorder such as cerebral infarct and encephalorrhagy, and cerebral circulation disorder. However, diseases to which hematopoietic factors of the present invention, rhM-CSF, rhG-CSF and rhEPO are applied are not limited to diseases in which the above-mentioned cholinergic nerve cell is involved. In more detail, while mβNGF has the limited activity on central nervous cell system such as peripheral sympathetic nerve, sensory nerve and cerebral cholinergic nerve, rhM-CSF, rhG-CSF and rhEPO of the present invention are originally factors on blood cell system cells, are considered to have the possibility of acting as a survival promoting factor on wider kinds of cerebral nerve cells, and this is considered to be a main cause for exerting the excellent cell survival prolonging activity. For this reason, rhM-CSF, rhG-CSF and rhEPO are promising as a drug for preventing or curing not only diseases associated with cholinergic nerve cells but also a wide range of diseases due to the cerebral function disorder such as Parkinson's disease which is caused by denaturation and shedding of dopaminergic

nerve cells in cerebral basal ganglia nigra, Huntington's chorea which is caused by denaturation and shedding of GABAergic nerve cell of corpus striatum (caudate nucleus, putamen) in cerebral basal ganglia.

[0029]

[Examples] Examples regarding preparations are illustrated below.

Example 1

8μg of erythropoietin and distilled water for injection were aseptically prepared into a solution at the above-mentioned compositional ratio to a total amount of 2ml, and the solution was dispensed into a vial and sealed.

[0030]

Example 2

8μg of erythropoietin and distilled water for injection were aseptically prepared into a solution at the above-mentioned compositional ratio to a total amount of 2ml, and the solution was dispensed into a vial, lyophilized and sealed.

[0031]

Example 3

16μg of erythropoietin and distilled water for injection were aseptically prepared into a solution at the above-mentioned compositional ratio to a total amount of 2ml, and the solution was dispensed into a vial and sealed.

[0032]

Example 4

16μg of erythropoietin and distilled water for injection

were aseptically prepared into a solution at the above-mentioned compositional ratio to a total amount of 2ml, and the solution was dispensed into a vial, lyophilized and sealed.

[0033] Example 5

8μg of erythropoietin, 5mg of human serum albumin and distilled water for injection were aseptically prepared into a solution at the above-mentioned compositional ratio to a total volume of 2 ml, and the solution was dispensed into a vial and sealed.

[0034] Example 6

8μg of erythropoietin, 5mg of human serum albumin and distilled water for injection were aseptically prepared into a solution at the above-mentioned compositional ratio to a total volume of 2 ml, and the solution was dispensed into a vial, lyophilized and sealed.

[0035] Example 7

16μg of erythropoietin, 5mg of human serum albumin and distilled water for injection were aseptically prepared into a solution at the above-mentioned compositional ratio to a total volume of 2 ml, and the solution was dispensed into a vial and sealed.

[0036] Example 8

16μg of erythropoietin, 5mg of human serum albumin and distilled water for injection were aseptically prepared into a solution at the above-mentioned compositional ratio to a total volume of 2 ml, and the solution was dispensed into a vial, lyophilized and sealed.

[0037] Example 9 to 12

According to the same manners as those of Examples 5 to 8 without using 5mg of dextran 40 in place of human serum albumin in Examples 5 to 8, injectables were prepared.

[0038] Example 13

5g of D-mannitol, 1mg of erythropoietin and 100mg of human serum albumin were aseptically dissolved into 100mg of distilled water for injection to obtain an aqueous solution, and each 1ml of the solution was dispensed into a vial, lyophilized and sealed.

[0039] Example 14

0.5mg of erythropoietin and 1g of sorbitol were aseptically dissolved in 50ml of a 0.05M-phosphate buffer at pH 7.0, to prepare an aqueous solution, and each 0.5ml of the solution was dispensed into a vial, lyophilized and sealed. Separately, a 0.1%-methylcellurose aqueous solution was aseptically prepared, and each 1ml of the solution was dispensed into an ampoule to obtain a solution for dissolution.

[0040] Example 15

75μg/ml of purified human G-CSF (10mM-phosphate buffer pH 7.0) and 15 mg/ml of D-mannitol were dissolved in distilled water for injection to 0.3ml, and the solution was filtration-sterilized with a membrane filter having a pore size of 0.22 μm. The resulting solution was filled into a sterilization-treated vial, half plugged with a similarly sterilization treated rubber, and subsequently secured by winding with an aluminium cap to obtain a solution preparation for injection. This solution preparation for injection is

stored in a cold dark place at 10°C or lower.

[0041] Example 16

An osmotic pressure ratio of 50 μg/ml of purified human G-CSF (10mM-phosphate buffer pH 7.0) was adjusted to 1 with NaCl, and filtration-sterilized with a membrane filter having a pore size of 0.22 μm. The resulting solution was filled into a sterilization-treated vial, plugged with a similarly sterilization-treated rubber, and subsequently secured by winding with an aluminium cap to obtain a solution preparation for injection. This solution preparation for injection is stored in a cold dark place at 10°C or lower.

[0042] Example 17

An osmotic pressure ratio of 100μg/ml of purified human G-CSF (10mM-phosphate buffer pH 7.0) was adjusted to 1 with NaCl, and filtration-sterilized with a membrane filter having a pore size of 0.22 μm. The resulting solution was filled into a sterilization-treated vial, plugged with a similarly sterilization-treated rubber, and subsequently secured by winding with an aluminium cap to obtain a solution preparation for injection. This solution preparation for injection is stored in a cold dark place at 10°C or lower.

[0043] Example 18

HSA and D-mannitol were added to 50μg/ml of purified human G-CSF (10mM-phosphate buffer pH 7.0) to dissolve them to the concentrations of 10 mg/ml and 50mg/ml, respectively, and filtration-sterilized with a membrane filter having a pore size of 0.22 μm. The resulting solution was filled into a

sterilization-treated vial, plugged with a similarly sterilization-treated rubber, and subsequently secured by winding with an aluminium cap to obtain a solution preparation for injection. This solution preparation for injection is stored under the temperature conditions at room temperature or lower, and is used by diluting with distilled water for injection upon use.

[0044] Example 19

Gelatin and D-mannitol were added to 100μg/ml of purified human G-CSF (10mM-phosphate buffer pH 7.0) to dissolve them to the concentrations of 10mg/ml and 50mg/ml, respectively, and filtration-sterilized with a membrane filter having a pore size of 0.22 μm. The resulting solution was filled into a sterilization-treated vial, half plugged with a similarly sterilization-treated rubber, and subsequently secured by winding with an aluminium cap to obtain a solution preparation for injection. This solution preparation for injection is stored under the temperature condition at room temperature or lower, and is used by diluting with distilled water for injection upon use.

[0045] Example 20

75μg/ml of purified human M-CSF (10mM-phosphate buffer pH 7.0) and 15mg/ml of D-mannitol were dissolved into distilled water for injection to 0.3ml, and filtration-sterilized with a membrane filter having a pore size of 0.22 μm. The resulting solution was filled into a sterilization-treated vial, half plugged with a similarly sterilization-treated rubber, and

subsequently secured by winding with an aluminium cap to obtain a solution preparation for injection. This solution preparation for injection is stored in a cold dark place at 10°C or lower.

[0046] Example 21

An osmotic pressure ratio of 50 μg/ml of purified human M/CSF (10mM-phosphate buffer pH 7.0) was adjusted to 1 with NaCl, and filtration-sterilized with a membrane filter having a pore size of 0.22 μm. The resulting solution was filled into a sterilization-treated vial, plugged with a similarly sterilization-treated rubber, and subsequently secured by winding with an aluminium cap to obtain a solution preparation for injection. This solution preparation for injection is stored in a cold dark place at 10°C or lower.

[0047]

[Effect of the invention] A preventing or curing drug containing rhM-CSF, rhG-CSF or rhEPO as an active ingredient of the present invention has the excellent cell survival prolonging activity and ChAT activating activity, and moreover, has the low side effect and, thus, is useful as a drug which is applied to various diseases due to the cerebral function disorder including Alzheimer's disease, Alzheimer type senile dementia or cerebrovascular dementia.

[Brief Description of the Drawings]

Fig. 1 is a graph showing a cell survival prolonging activity of rhEPO in an intracerebral in vivo model obtained by Experimental example [3]. In Fig. 1, longitudinal axis is

a survival ratio and horizontal axis is an experimental group respectively.

たパーセンテージをアセチルコリン作動性神経細胞の生存率とした。結果を図1に示す。なお、図中のカラムはMean±1S．D．であり、＊＊はp＜0．01を示し、また、EPO−Hは125I．U．／15μl／day投与群を、EPO−Lは12．5I．U．／15μl／day投与群をそれぞれ示す。

【0019】〔4〕コリンアセチルトランスフェラーゼ（ChAT）活性の測定及び蛋白質の定量
ChAT活性は、Fonnumの方法〔F．Fonnum, J．Neurochem．，24，407〜409（1975）〕に基づいて求めた。すなわち、上記の初代培養細胞あるいはSN6.10.2.2培養細胞をラバーポリスマンで回収した後、30μlの10mM−EDTA液中でホモジナイズし、更に最終濃度0．5％（v／v）Triton−X100を加えたものを酵素源とした。酵素反応溶液は300mM−NaCl、50mMリン酸ナトリウム緩衝液（pH7．4）、20mM−EDTAに基質として0．2mM〔1−$^{14}$C〕acetyl−CoA（アマシャム　ジャパン社より購入）及び8mM−Choline bromide及びアセチルコリンエステラーゼ阻害剤として0．1mM−Physostigmineを加えたものである。この反応溶液5μlを＊1．5mlのマイクロチューブ（エッペンドルフ社製、ドイツ）に入れ、酵素液サンプル2μlを加えて軽く振とう撹拌し、37℃で15分間反応させた。氷冷することによって反応を停止させ、マイクロチューブを液体シンチレーション用バイアルの中に入れて中身の反応混液を5mlの10mM−Phosphate bufferで洗いだした。このバイアルに10mg／mlのカリグノスト（Sodium Tetraphenylborate）を含む2mlのアセトニトリルと10mlのトルエン系シンチレーターを加え、1分間軽く振とうした。バイアルを10分間静置した後、液体シンチレーションカウンターによって$^{14}$Cで標識されたアセチルコリンの量を決定し、ChAT活性を求めた。

【0020】また、蛋白質の定量は、ローリー法〔Lowryら，J．Biol．Chem．，193，265〜275（1951）〕に従って行った。この目的で上述の酵素液サンプルの10μlを使用した。そして、これらの値を基に比活性、総蛋白量及び総ChAT活性をそれぞれ算出した。ChAT活性、タンパク質濃度及び生存率についてはt−testを行った。

【0021】

【表1】

| | n | ChAT比活性 (P moles/mg/min) (mean ±SD) | 相対ChAT比活性 a) | 総蛋白量 (μg/well) (mean±SD) | 相対総蛋白量 b) | 総ChAT活性 (P moles/hr/well) c) (mean±SD) | 相対総ChAT活性 d) |
|---|---|---|---|---|---|---|---|
| Control | 5 | 13.2±0.9 | 100 | 118.5±18.9 | 100 | 98.8±11.0 | 100 |
| mβNGF (ng/ml) 100 | 4 | 21.3±2.2 | 161 | 146.7±25.6 | 123 | 185.3±23.1 | 187 |
| hM-CSF (CFU/ml) 10 | 3 | 12.8±2.4 | 97 | 148.7±17.6 | 125 | 112.3±14.1 | 120 |
| 50 | 3 | 15.5±0.7 | 117 | 171.7±16.1 | 144 | 159.4±19.5 | 170 |
| 100 | 3 | 15.5±0.7 | 117 | 137.8± 2.9 | 115 | 128.1± 8.2 | 137 |
| hG-CSF (CFU/ml) 10 | 3 | 13.3±1.9 | 101 | 133.5±32.4 | 112 | 107.2±31.3 | 114 |
| 50 | 3 | 16.5±2.7 | 125 | 147.8±12.4 | 124 | 145.1±20.1 | 155 |
| 100 | 3 | 15.0±1.5 | 114 | 140.9±15.8 | 118 | 125.2± 4.0 | 133 |
| hEPO (IU/ml) 1 | 3 | 11.9±0.8 | 90 | 157.0±21.2 | 131 | 111.5± 8.1 | 119 |
| 5 | 3 | 15.9±2.3 | 120 | 149.5±20.5 | 125 | 140.3± 5.0 | 149 |
| 10 | 3 | 15.6±0.7 | 118 | 164.6±31.8 | 138 | 152.4±22.7 | 162 |

（注）

$$a：相対ChAT比活性＝\frac{（サンプルのChAT比活性）}{（Control　のChAT比活性）}\times 100$$

$$b：相対総蛋白量＝\frac{（サンプルの総蛋白量）}{（Control　の総蛋白量）}\times 100$$

c：総ChAT活性＝ChAT比活性×総蛋白量

$$d：相対総ChAT活性＝\frac{（サンプルの総ChAT活性）}{（Control　の総ChAT活性）}\times 100$$

【0022】

【表2】

| | n | ChAT比活性 (P moles/mg/min) | 相対ChAT比活性[a)] | 総蛋白量 μg/well | 相対総蛋白量[b)] | 総ChAT活性 (P moles/hr/well)[c)] | 相対総ChAT活性[d)] |
|---|---|---|---|---|---|---|---|
| Control | 1 | 20.2 | 100 | 147.1 | 100 | 179.2 | 100 |
| hM-CSF (CFU/ml) 50 | 1 | 34.1 | 168 | 157.1 | 107 | 322.2 | 180 |
| hG-CSF (CFU/ml) 50 | 1 | 32.6 | 161 | 146.9 | 100 | 283.5 | 158 |
| hEPO (IU/ml) 10 | 1 | 36.5 | 180 | 142.6 | 97 | 311.7 | 174 |

（注）a、b、c及びdは、表1の場合と同じである。

【0023】上記表1に示す結果から明らかなように、中隔野神経細胞の初代培養系に本発明のｒｈＭ－ＣＳＦ、ｒｈＧ－ＣＳＦ又はｒｈＥＰＯを表中に示すＤｏｓｅで添加したときの５日後のＣｈＡＴ比活性、総蛋白量及び総ＣｈＡＴ活性を調べた結果は、ｒｈＭ－ＣＳＦの場合にはＣｏｎｔｒｏｌに対してＤｏｓｅ：１０ＣＦＵ／ｍｌ、５０ＣＦＵ／ｍｌ及び１００ＣＦＵ／ｍｌで総蛋白量をそれぞれ２５％、４４％及び１５％増加させ、ｒｈＧ－ＣＳＦの場合にはＣｏｎｔｒｏｌに対してＤｏｓｅ：１０ＣＦＵ／ｍｌ、５０ＣＦＵ／ｍｌ及び１００ＣＦＵ／ｍｌで総蛋白量をそれぞれ１２％、２４％及び１８％増加させ、また、ｒｈＥＰＯの場合にはＣｏｎｔｒｏｌに対してＤｏｓｅ：１ＩＵ／ｍｌ、５ＩＵ／ｍｌ及び１０ＩＵ／ｍｌで総蛋白量をそれぞれ３１％、２５％及び３８％増加させせた。これらの値は、対照として実験したｍβＮＧＦ（１００ｎｇ／ｍｌ）の場合の効果２３％と同等あるいはそれ以上の結果を示すものであり、本発明で使用する造血因子類が神経栄養因子のｍβＮＧＦと類似の作用を発揮し、初代培養神経細胞の生存を促進することが判明した。

【0024】また、ＣｈＡＴ比活性に関しては、Ｃｏｎｔｒｏｌに対して、ｒｈＭ－ＣＳＦがＤｏｓｅ：５０ＣＦＵ／ｍｌ及び１００ＣＦＵ／ｍｌでそれぞれ１７％増加させ、ｒｈＧ－ＣＳＦがＤｏｓｅ：１０ＣＦＵ／ｍｌ、５０ＣＦＵ／ｍｌ及び１００ＣＦＵ／ｍｌでそれぞれ１％、２５％及び１４％増加させ、また、ｒｈＥＰＯがＤｏｓｅ：５ＩＵ／ｍｌ及び１０ＩＵ／ｍｌでそれぞれ２０％及び１８％増加させせた。更に、総ＣｈＡＴ活性については、Ｃｏｎｔｒｏｌに対して、ｒｈＭ－ＣＳＦがＤｏｓｅ：１０ＣＦＵ／ｍｌ、５０ＣＦＵ／ｍｌ及び１００ＣＦＵ／ｍｌでそれぞれ２０％、７０％及び３７％増加させ、ｒｈＧ－ＣＳＦがＤｏｓｅ：１０ＣＦＵ／ｍｌ、５０ＣＦＵ／ｍｌ及び１００ＣＦＵ／ｍｌでそれぞれ１４％、５５％及び３３％増加させ、また、ｒｈＥＰＯがＤｏｓｅ：１ＩＵ／ｍｌ、５ＩＵ／ｍｌ及び１０ＩＵ／ｍｌでそれぞれ１９％、４９％及び６２％増加させた。これらの結果から、本発明で使用する造血因子、ｒｈＭ－ＣＳＦ、ｒｈＧ－ＣＳＦ又はｒｈＥＰＯは、ＣｈＡＴ賦活作用においても優れた効果を発揮することが判明した。

【0025】更に、表２は、ＳＮ6.10.2.2細胞のＣｈＡＴ比活性、総蛋白量及び総ＣｈＡＴ活性に対する本発明のｒｈＭ－ＣＳＦ、ｒｈＧ－ＣＳＦ及びｒｈＥＰＯの影響を示すもので、この系では、総蛋白量がＣｏｎｔｒｏｌの場合と造血因子を添加した場合とでほとんど変化していないことからも分かるように、造血因子類の分化形質に対してのみの影響を単独で観察することができる。この実験の結果から明らかなように、ｒｈＭ－ＣＳＦ（５０ＣＦＵ／ｍｌ）、ｒｈＧ－ＣＳＦ（５０ＣＦＵ／ｍｌ）及びｒｈＥＰＯ（１０ＩＵ／ｍｌ）は、ＣｈＡＴ比活性をそれぞれ６１％、６８％及び８０％上昇させ、また、総ＣｈＡＴ活性をそれぞれ８０％、５８％及び７４％上昇させた。

【0026】また、図１に示したように、ｒｈＥＰＯ投与群では、対照群（生理食塩水、０．１％ＢＳＡ）に比べて片側性にＦｉｍｂｒｉａ－Ｆｏｒｎｉｘ切断したラットの中隔野におけるＡｃｈＥ陽性細胞の生存率が有意に改善された。片側性にＦｉｍｂｒｉａ－Ｆｏｒｎｉｘ切断したラットの中隔野で、ｒｈＥＰＯのアセチルコリン作動性神経細胞に対するｉｎ ｖｉｖｏの効果が確認されたことは、実際の臨床的展開を考える上で意義深い。

【0027】

【作用】以上の実験から明らかなように、本発明のｒｈＭ－ＣＳＦ、ｒｈＧ－ＣＳＦ及びｒｈＥＰＯは、元々血液細胞系に対して作用を有するものとして発見された造血因子であるが、中枢神経細胞系に対してもｍβＮＧＦと同様の活性を有することが判明した。すなわち、マウス中隔野神経細胞のｉｎ ｖｉｔｒｏ初代培養において総蛋白量を顕著に増加させると共にＣｈＡＴ比活性や総ＣｈＡＴ活性を増加させ、また、マウス中隔野由来コリン作動性神経細胞株ＳＮ6.10.2.2細胞においてもＣｈＡＴ比活性や総ＣｈＡＴ活性を増加させ、従って、これらの造血因子、ｒｈＭ－ＣＳＦ、ｒｈＧ－ＣＳＦ及びｒｈ

【図1】